Supporting Information

A Steroid-Conjugated Magnetic Resonance Contrast Agent for In vivo Imaging of Cell Signaling

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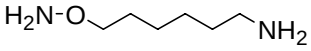
Experimental Procedure:

General Methods. Unless otherwise noted, materials and solvents were obtained from commercial suppliers and used without further purification. All organic reactions were performed under an atmosphere of N_2 in oven-dried glassware unless otherwise stated. Thin-layer chromatography was performed on Merck 60F 254 silica gel plate. Visualization of the developed chromatogram was performed by CAM stain and platinum stain. Flash chromatography was carried out using Fisher Grade 60Å 230-400 mesh silica gel. Organic extracts were dried over $MgSO_4$ and were concentrated using a Büchi rotary evaporator under reduced pressure. NMR spectra were obtained on a Varian Inova spectrometer at 500MHz and a Varian Mercury spectrometer at 400MHz. NMR chemical shifts are reported in ppm and referenced to residual protonated solvent. Mass spectrometry samples were analyzed using electrospray (ESI) ionization by Varian 1200L Quadrupole MS system. ICP-MS was performed on a PQ ExCell Inductively Coupled Plasma Mass Spectrometer. Elemental analysis was performed by Desert Analytics (Tucson, AZ). HPLC analyses were performed on a Varian Prostar 500 (for analysis)/120 (for preparation) system. The longitudinal water proton relaxation rate at 59.97 MHz was measured by using a Bruker mq60 NMR Analyzer (Bruker Canada, Milton, Ont. Canada)

Synthesis

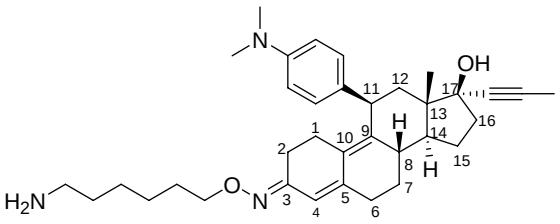
O-(6-Amino-hexyl)-hydroxylamine (2)



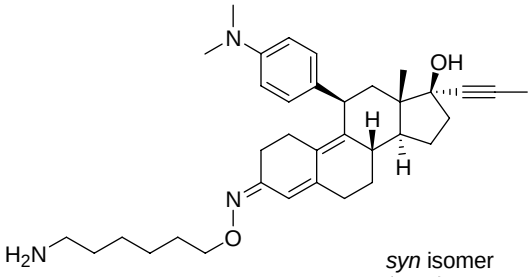
2 was synthesized as previously described.[1] Three steps from 1,6-dibromohexanol (commercially available from Aldrich), overall 58% yield, white solid

^{1}H NMR (400MHz, D_2O): δ =3.90 (2H, $NOCH_2$, t, *J*= 6.4), 2.82 (2H, $C\underline{H}_2NH_2$, t, *J*= 7.2), 1.51 (4H, m), 1.24 (4H, m) ; MS m/z 133.1 $[M+H^+]^+$

11-(4-Dimethylamino-phenyl)-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,13,14,15,16,17-dodecahydro-cyclopenta[a]phenanthren-3-one O-(6-amino-hexyl)-oxime (3)



anti isomer (60%)



syn isomer (40%)

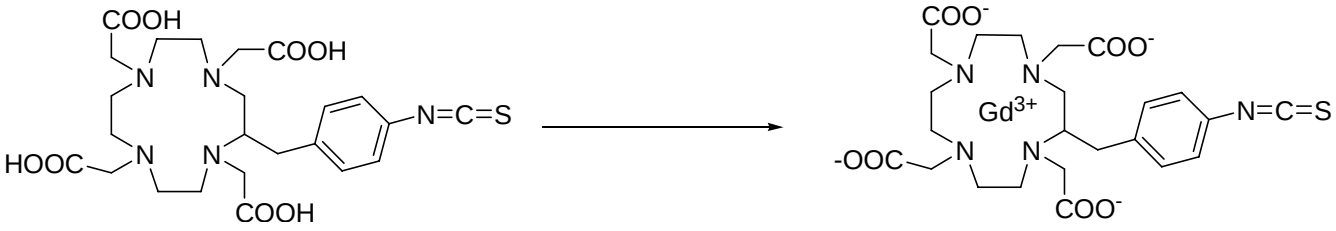
To a stirred solution of **RU-486** (229mg, 0.54mmol) in Ethanol (5mL) was added **2** (85mg, 0.64mmol). The mixture was stirred for 2h at room temperature and concentrated in vacuo. The residue was purified by column chromatography using 1% to 25% of methanol solvent gradient in dichloromethane to obtain pale yellow solid. (159mg, 54%)

[1] Heikki Mikola, E. H., Introduction of aliphatic amino and hydroxy groups to keto steroids using O-substituted hydroxylamines, *Bioconjugate Chemistry* **1992**, *3*, 182-186.

The product consists of 3 : 2= anti : syn isomers, however this isomeric mixture was used without further purification.

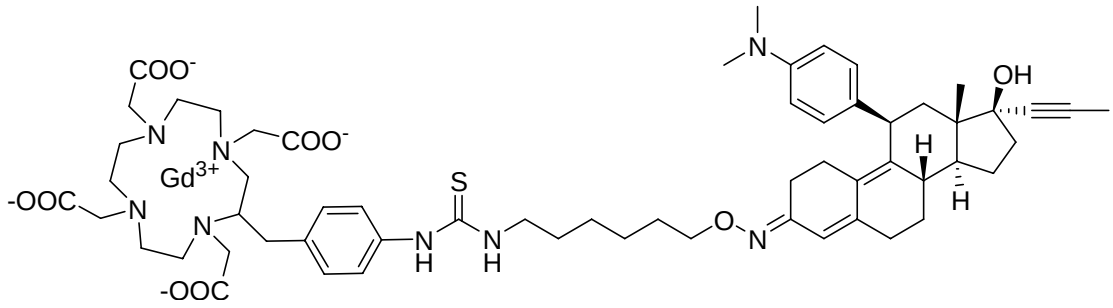
^{1}H NMR (CD_3OD): δ = 7.04 (2H, d, *J*=7.5, aryl), 6.70 (2H, d, *J*=8, aryl), 6.37 (0.4H, s, syn 4-H), 5.78 (0.6H, s, anti 4-H), 4.29 (1H, d, *J*=5 ,11-H), 4.00 (2H, m, $NOCH_2$), 2.8-2.9 (8H, m, 2×N-CH_3, $C\underline{H_2}NH_2$), 2.2-2.6 (7H, m, 1-,2-,6-,8-H), 1.8-2.1 (11H, m, 12-,14-,15-,16-H, 2×CH_2-linker), 1.82 (3H, s, C≡CCH_3) 1.2-1.6 (6H, m, 7-H, 2×CH_2-linker), 0.5 (3H, s, 13-CH_3); MS m/z 544.4 $[M+H^+]^+$

(4)



To a solution of the free ligand (103mg, 0.15mmol; commercially available from Macrocyclics) in water (3mL) was added gadolinium(III) chloride (60mg, 0.16mmol). The pH of solution was being monitored and kept between 6.0 and 6.5 using 0.1M NaOH solution. When there was no longer change in the pH of the solution, the reaction mixture was then freeze dried. The dried residue was purified by HPLC using aquasil C18 column to obtain white solid (87mg, 83%). MS m/z 704.2 $[M-H^+]^-$ Gd isotope pattern centered at 704.2

(1)



A solution of **3** (87mg, 0.12mmol), triethylamine (25μL, 0.18mmol), and **4** (72mg, 0.13mmol) in DMSO (3mL) was stirred overnight at room temperature. After the complete disappearance of **3** on TLC, the mixture was freeze dried. The residue was redissolved in water (5mL) and filtered through a 0.2μm Nylon filter to get rid of any remaining steroid compound **3**. The filtrate was purified by HPLC using Vydac denali C18 small pore column to obtain white solid (97mg, 65%). The fractions were analyzed by analytic LC-MS, and due to the paramagnetic nature of Gd(III) ion, it was unable to characterize **1** by NMR spectroscopy. ESI-MS m/z 1248.5 $[M\text{-}H^+]^-$ Gd isotope pattern centered at 1248.5 . Anal. Calcd for $C_{59}H_{78}N_8O_{10}Gd\ S^-Na^+\cdot H_2O$: C 54.95, H 6.25, N 8.69; Found C 55.15, H 6.18, N 8.31.

Biological Studies

Transcription assay with GAL4-UAS system

Rat1 fibroblasts (grown in DMEM, 10% fetal bovine serum) were seeded in triplicate in six well plates (2 x 10^5 cells/well) and transfected the following day using Lipofectamine Plus (Gibco) following the manufacturer's protocol. Transfections contained the UAS-

reporter plasmid (250 ng, pGene-lacZ, Invitrogen), and the Gal4-Progesterone receptor (250 ng, pSwitch, Invitrogen). The total amount of DNA per well was adjusted to 1 μg by adding pcDNA 3.1 vector as carrier. After removing the DNA complexes, cells were fed with fresh medium containing 10^{-8} - 10^{-6} M RU486 (from a 1 mM stock dissolved in 100% Ethanol) or **1** (from a 0.5 mM stock dissolved in 1:1 (v/v) 100% Ethanol:DMSO) (Figure 1). Twenty-four hours after transfection, cells were harvested to determine β-galactosidase activity (chemiluminescent; Roche) by luminometry. To calculate relative fold change, all values were normalized to the pGene-lacZ (UAS-lacZ) minus RU486 control sample.

Progesterone response element (PRE)-luciferase transcriptional activation.

T47D breast cancer epithelial cells (American Type Culture Collection, Mananssa, VA) were cultured in phenol-red free RPMI (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), and 1% antimycotic/antibiotic (Invitrogen, Carlsbad, CA) and incubated at 37° C, 5% CO_2 . Cells were plated 1 day before transfection in 24 well plates and transiently transfected in Opti-MEM (Life Technologies) with PRE-luciferase (a kind gift from Ken Korach, NIEHS, NIH). Cells were then treated with either serum free media and vehicle (DMSO), progesterone, RU486, or **1** for 24h. To measure luciferase production, cells were lysed in GME buffer [25mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4mM EGTA, 1mM dithiothreitol, and 1% Triton X-100] and lysates were added to assay buffer (GME buffer, 16.5 mM KPO4 , 2.2 mM ATP, and 1.1 mM dithiothreitol). Luciferase activity was measured for 30 sec using an AutoLumat (Berthold Technologies Co.,Oak Ridge,TN). A separate protein

determination using the BCA kit (Pierce) was used to normalize protein levels that might differ from treatment with hormone.

Progesterone Receptor Binding Assay

The progesterone receptor A ligand binding domain (aa 675-933) fused to GST (PR-LBD-GST;80 nM), a fluorescently-tagged progesterone ligand (fluoromone™ green PL; 4 nM), and either progesterone (1 uM), RU486 (several concentrations), or **1** (several concentrations) were incubated in PR screening buffer with 4 mM DTT in a total volume of 100 uL for 1 hour at RT according to the manufacturer protocol (Invitrogen, Carlsbad, CA). Each sample was performed in at least triplicate using the Beacon 2000 fluorescence polarization analyzer (Invitrogen) located in the Northwestern University Keck Facility. The machine was used in static mode, batch blank, no delay, average of 1 read per cycle, at 22 °C. An average of 3 samples containing only buffer and PR-LBD-GST with no fluorescent PL was used as the blank to eliminate background signal from the protein or buffer. A sample with no competitor was used to determine 100% binding capacity of the PR-LBD-GST for the PL ligand.

Mammary cellular uptake of **1** measured by ICP-MS.

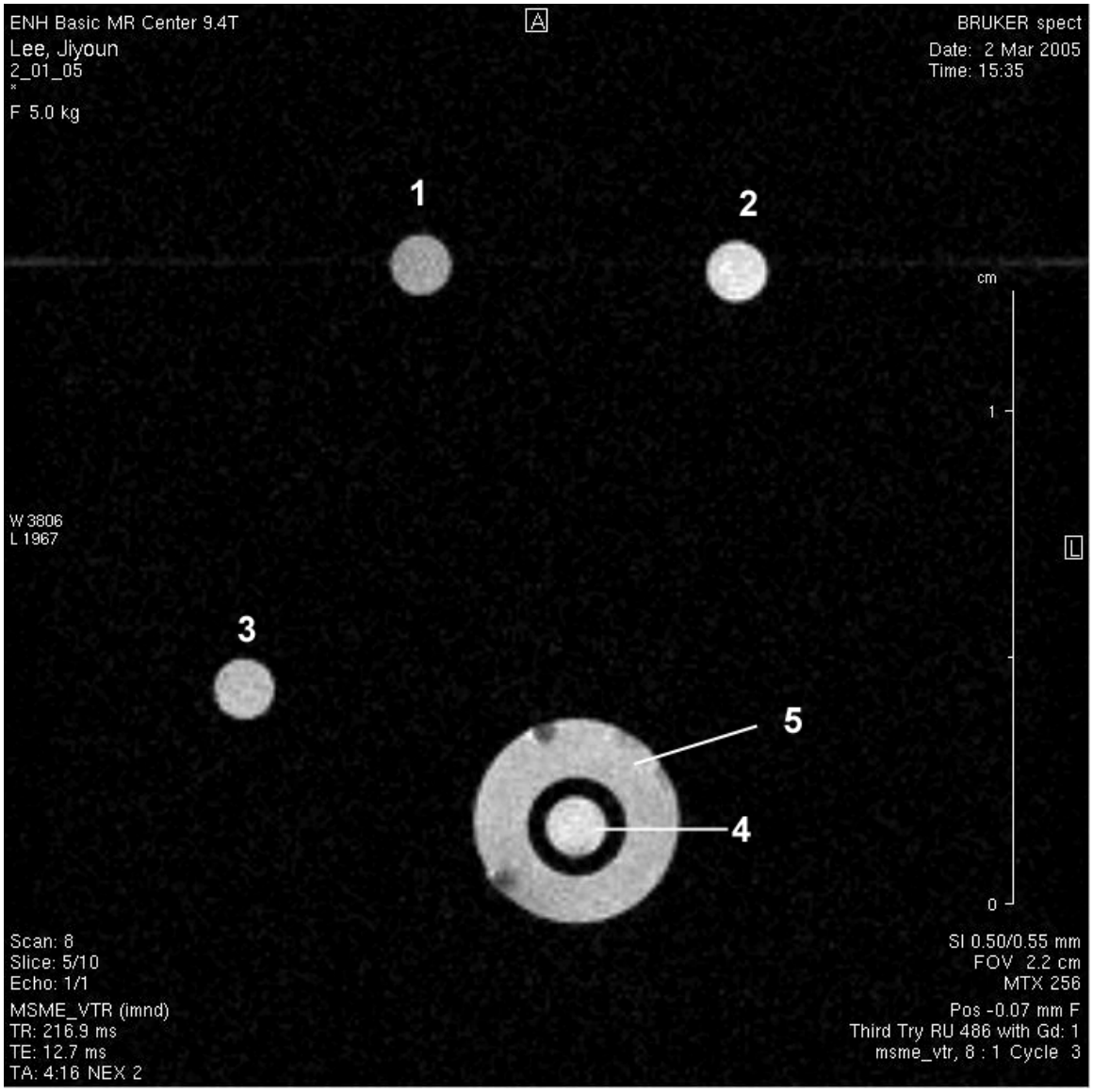
Progesterone receptor (PR) positive cells, T47D, and progesterone receptor negative cells (MDA-MB-231) were used to determine uptake efficiency of **1** into hormone receptor expressing cells. MDA-MB-231 breast cancer epithelial cells (American Type Culture Collection, Mananssa, VA) were cultured in phenol-red free DMEM/F12 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10 μg/mL insulin, 5%

charcoal dextran stripped FBS (Cellgro), 1% glutamax, and 1% antimycotic/antibiotic (Invitrogen, Carlsbad, CA) and incubated at 37° C, 5% CO_2 . Cells were plated into 12 well dishes and the next day moved into serum free medium for 24 hours before treatment with RU486 or **1**. To determine the dose of **1** necessary to accumulate in the cells the following doses were incubated with cells for 24 hrs: 0, 0.1, 1, 10, 50, and 100 μM. In order to determine the optimal incubation time 100 μM RU-486 was incubated with the cells for the following time periods: 0, 1, 2, 4, 16, and 24 hours. After incubation, each sample was rinsed three times with PBS (2-3ml) maintained at ambient temperature to insure removal of extra cellular and unbound contrast agent. The cells were exposed to 250ul of 0.25% trypsin and harvested. The cells were counted using hemacytometer and checked for viability using a trypan blue essay. The counted cells were then digested in nitric acid at 80^{o}C for 4hrs, and analyzed by ICP-MS.

MRI images of the cells incubated with 1

The cells were incubated and washed as the method described above. (50μM of **1** and RU-486 for 16 hrs) An average of 1,500,000 cells were loaded into NMR tube coaxial inserts (catalog number WGS-5BL, Wilmad, NJ) as trypsin suspensions. MR studies were performed on a General Electric/Bruker, Omega 400 WB imaging spectrometer fitted with Accustar shielded gradients at ambient temperature (20°C). Spin-lattice relaxation time was measured using an inversion recovery pulse sequence. Images were aquired using a spin-echo imaging pulse sequence with a short recycle time (TR) of 300ms, echo delay time (TE) of 10ms.

Figure 1. MRI picture of the cells incubated with RU-486 and **1**



1. PR + with RU-486, T_1=3.18±0.11s
2. PR + with **1,** T_1=2.06±0.16s
3. PR – with RU-486, 2.90±0.11s
4. PR – with **1,** T_1=2.30±0.30s
5. Media, T_1=3.03±0.06s